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PURIFICATION AND CHARACTERIZATION OF TWO ESSENTIAL CYTOCHROMES OF THE THIOSULPHATE-OXIDIZING MULTI-ENZYME SYSTEM FROM *THIOBACILLUS* A_2 (*THIOBACILLUS VERSUTUS*)

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Four *c*-type cytochromes were purified by several procedures including chromatography on DEAE-Sepharose CL-6B, Phenyl-Sepharose CL-4B and Sephadex G-75, G-100 and G-200 and chromatofocusing. Cytochrome *c*-551 had a p*I* value of 5.2 and an M_r of 260000 consisting of six non-covalently bound polypeptides each with an M_r of 43000, and contained four to five haems. Cytochrome *c*-552.5 had a p*I* value of 4.8 and an M_r of 56000 consisting of two polypeptides with the same M_r 29000, and contained two haems. Cytochromes *c*-551 and *c*-552.5 were reduced by ascorbate to about 70 and 60% of the fully dithionite-reduced values, respectively, and both were essential components in the thiosulphate-oxidizing multi-enzyme system (other components of the system were 'enzyme A', 'enzyme B' and sulphite: cytochrome *c* oxidoreductase). These two cytochromes functioned as electron carriers and effectors in the oxidation of thiosulphate. Some evidence suggested that cytochrome *c*-551 might be a specialized electron transfer component for sulphonate-sulphur oxidation. Both cytochromes could be reduced by thiosulphate in the presence of enzymes A and B. Cytochrome *c*-550 (basic) and cytochrome *c*-550 (acidic) were small proteins with M_r 15000 and 14000 and p*I* values of over 8 and 5, respectively. Their physiological role is uncertain.

Introduction

c-Type cytochromes are generally regarded as essential components in the chemolithotrophic oxidation of thiosulphate from the facts that they are the primary acceptors for electrons from the substrate oxidation in nearly all of the thiobacilli except probably *Thiobacillus denitrificans* [1], and the contents of *c*-type cytochromes are much higher in autotrophically grown than heterotrophically grown *Thiobacillus* A_2 and *Thiobacillus novellus* [2,3]. This type of study was mainly carried out with either crude extract or membrane systems. Although numerous workers have reported the identification and purification of the *c*-type cytochromes from thiobacilli over more than 20 years [1,4], the investigations were limited to the small, basic *c*-type cytochromes resembling the mammalian cytochromes and provided little information related directly to the role of *c*-type cytochromes in thiosulphate oxidation.

Recently, we reported [5,6] that the thiosulphate-oxidizing system of *Thiobacillus* A_2 was a multi-enzyme complex composed of up to five soluble components, namely enzyme A, enzyme B, cytochrome *c*-552.5, cytochrome *c*-551 and sulphite: cytochrome *c* oxidoreductase, apart from membrane particles which functioned as electron

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chain components, and could be replaced by mammalian cytochrome *c* and cytochrome oxidase. Enzyme A and enzyme B were highly purified and characterized and an investigation on sulphite : cytochrome *c* oxidoreductase is to be published elsewhere [6]. The present communication describes the purification and some properties of cytochrome *c*-552.5 and cytochrome *c*-551 as well as two small cytochrome *c*-550. The role of cytochrome *c*-552.5 and cytochrome *c*-551, functioning as special redox carriers or effectors, in the thiosulphate-oxidizing system was also investigated and discussed.

Materials and Methods

Growth of the organism and preparation of cell free extract. These were described previously [5–7].

Purification of enzyme A and enzyme B. These were as stated before [6].

Enzyme assay. Thiosulphate : cytochrome *c*-oxidoreductase activity was measured by following the reduction of cytochrome *c* (horse heart III) essentially as described previously [5–7].

The reaction mixture contained, unless otherwise stated, 2 μM $Na_2S_2O_3$, 1 mg horse heart cytochrome *c*, 0.05–0.1 mg enzyme A, 0.03–0.06 mg enzyme B, 0.02–0.04 mg cytochrome *c*-552.5, 0.04–0.08 mg cytochrome *c*-551 and 35–45 μmol Tris-HCl (pH 7.3) to give a final volume of 1 ml.

Activities of cytochromes *c*-551 and *c*-552.5 in the thiosulphate : cytochrome-*c*-oxidoreducing system were assayed as above except that cytochrome *c*-552.5 was omitted for assay of cytochrome *c*-551 and vice versa. It was virtually impossible to evaluate the activities of these two cytochromes quantitatively in the course of the purifications mainly due to the facts that (a) the two cytochromes were not completely separated from each other in the 0.35 M NaCl (I) and 0.35 M NaCl (II) fractions and very small amounts (0.002 mg or less) of cytochrome *c*-552.5 (or cytochrome *c*-551) in the reaction mixture for assay of cytochrome *c*-551 (or for assay of cytochrome *c*-552.5) greatly enhanced the activity and (b) both of them were probably activators or electron carriers to assist the whole enzyme system rather than functioning as enzymes (see Results for further description).

Thiosulphate-oxidizing activity was measured polarographically with a Clark oxygen electrode as described previously [3,6]. The reaction mixture was essentially the same as the spectrophotometric method described above except that higher amounts of the two enzymes and the two cytochromes were used as indicated, and bovine heart cytochrome oxidase (five units) was added.

Terminology. Thiosulphate-oxidizing multi-enzyme system (or complex) means the mixture containing enzyme A, enzyme B, cytochrome *c*-552.5, cytochrome *c*-551 and sulphite : cytochrome *c* oxidoreductase or means the mixture containing enzyme A, enzyme B and either both or one of the two cytochromes, which are able to start the reaction either spectrophotometrically in the presence of thiosulphate and horse heart cytochrome *c* (also called thiosulphate : cytochrome *c* oxidoreductase system) or polarographically in the additional presence of cytochrome oxidase. Sulphite : cytochrome *c* oxidoreductase was not normally included in the reaction mixture due to the fact that this enzyme was not essential in the assay of thiosulphate oxidation activity, but which did not mean the enzyme was less important than others of the multi-enzyme system in vivo (Lu and Kelly, unpublished data).

Preliminary purification of c-type cytochromes. As reported before, the crude extract of *Thiobacillus* A_2 was fractionated by ammonium sulphate to obtain A 65% fraction [5] which was then resolved into five major fractions by chromatography on DEAE-Sepharose CL-6B [5–7], where 0.35 M NaCl (I), 0.35 M NaCl (II), zero-NaCl and 0.2 M NaCl fractions contained cytochromes *c*-551, *c*-552.5, *c*-550 (basic) and *c*-550 (acidic), respectively. From this stage these *c*-type cytochromes were further purified and detailed in the present paper.

Purification of cytochrome c-551

Gel filtration on Sephadex G-100. The 0.35 M NaCl (I) fraction (550 mg) from the DEAE-Sepharose CL-6B chromatography was loaded on to the bottom of a column (3.2 × 89.5 cm) of Sephadex G-100 equilibrated with 50 mM Tris buffer (pH 7.3), and eluted upwards with the same buffer at 4°C and a flow rate of 30 ml · h^{-1}. Cytochrome *c*-551, coming out at the void volume (Fig. 1a) was combined and concentrated by salt out with ammonium sulphate. The concentrated material was

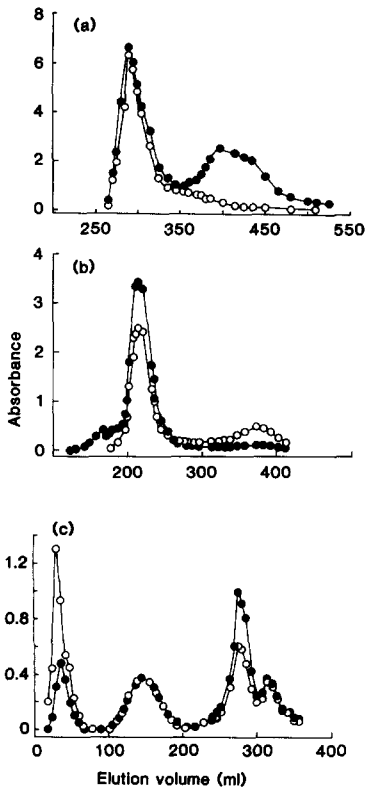

Fig. 1. Purification of cytochrome *c*-551 (see Materials and Methods for previous procedures and detail). ●, protein (A_{280}); ○, cytochrome c_{551} (A_{416}). (a) Elution profile of 0.35 M NaCl (I) fraction on Sephadex G-100. Void volume was 280 ml. Sulphite: cytochrome *c* oxidoreductase and enzyme B activities, eluted at the fractions from 310–390 ml and from 390–470 ml, respectively, are not shown. Fractions containing cytochrome *c*-551 from 275 ml to 305 ml were pooled and named G-100 fraction. (b) Elution pattern of the G-100 fraction on Sephadex G-200. Void volume was 165 ml. (c) Elution profile of the G-100 fraction on Phenyl-Sepharose CL-4B. Cytochrome *c*-551 existed in all the protein peaks except the first one which contained an unidentified *c*-type cytochrome.

called the G-100 preparation. The reasons for using Sephadex G-100 for this step instead of the Sephadex G-200 used previously [6] were that the exclusion of the cytochrome (M_r approx. 300 000) from Sephadex G-100 greatly decreased the tailing of the cytochrome and gave better separation of sulphite: cytochrome *c* oxidoreductase from the cytochrome.

Gel filtration on Sephadex G-200. The G-100 preparation (150 mg) was applied to the bottom of a column (2.6 × 88.5 cm) of Sephadex G-200. Procedures of the chromatography and concentration were the same as stated above except that a flow rate of 18 ml · h^{-1} was used (Fig. 1b).

Hydrophobic interaction chromatography. The G-100 preparation (112 mg) was brought to about 15% of saturation with $(NH_4)_2SO_4$ and loaded on to a column (2.6 × 6 cm) of Phenyl-Sepharose CL-4B equilibrated with 50 mM Tris (pH 7.3), containing 10% of saturation with $(NH_4)_2SO_4$ at 4°C. The column was then eluted with one bed volume of the same buffer at a flow rate of 30 ml · h^{-1}, followed by a linear gradient of decreasing ammonium sulphate concentration, which was produced from two 150 ml vols. of 50 mM Tris (pH 7.3), one of which contained $(NH_4)_2SO_4$ (10% of saturation) (Fig. 1c). Fractions belonging to the same protein peak were combined and concentrated by ultrafiltration through an Amicon PM10 membrane under N_2 pressure.

Chromatofocusing. 25 mg of concentrated cytochrome *c*-551 from gel filtration on Sephadex G-200 was loaded on to a column of Polybuffer exchangers PBE 94 equilibrated with 25 mM piperazine-HCl buffer (pH 5.4), at 4°C. The column was eluted with 10% polybuffer 74-HCl (pH 3.5), at a flow rate of 24 ml · h^{-1}. One major protein peak was eluted at pH 3.9–3.7, pooled and concentrated by ultrafiltration. When the effluent reached pH 3.5, the red protein remaining on the top part of the column was removed and then dissociated from the exchanger by adding a few ml of 1 M NaCl. The protein solution was concentrated by salt out with $(NH_4)_2SO_4$, and then desalted by Sephadex G-25 if required.

Purification of cytochrome c-552.5

Gel filtration on Sephadex G-100. 420 mg of 0.35 M NaCl (II) fraction from DEAE-Sepharose CL-6B chromatography was loaded on to a 3.2 × 88.5 cm column of Sephadex G-100 and eluted as described for that of cytochrome *c*-551 above. The cytochrome was recovered as a single coincident peak for absorbance at 280 and 416 nm in the fractions eluted between 350–450 ml. Combined fractions were concentrated by ultrafiltration.

Chromatofocusing. The procedures were the same as that of cytochrome *c*-551 except that 120 mg protein sample was used.

Gel filtration of Sephadex G-75. Cytochrome

c-552.5 from gel filtration on Sephadex G-100 or chromatofocusing was further purified by chromatography on Sephadex G-75 with a column of 2.2 × 87 cm if required. The conditions of the chromatography were the same as that on Sephadex G-100 except a flow rate of 18 ml · h^{-1} was used.

Purification of cytochrome c-550 (basic). During the chromatography of the A 65% fraction on DEAE-Sepharose CL-6B, the fractions of the second half of the zero-NaCl elution, containing cytochrome *c*-550, were combined and concentrated by salt out with $(NH_4)_2SO_4$. The concentrated fraction was applied on to a column (2.2 × 87 cm) of Sephadex G-75 and eluted as described above (Fig. 2a). Fractions containing cytochrome *c*-550 (with a ratio of A_{416}–A_{280} greater than 3.0) were pooled and concentrated by salt out with $(NH_4)_2SO_4$.

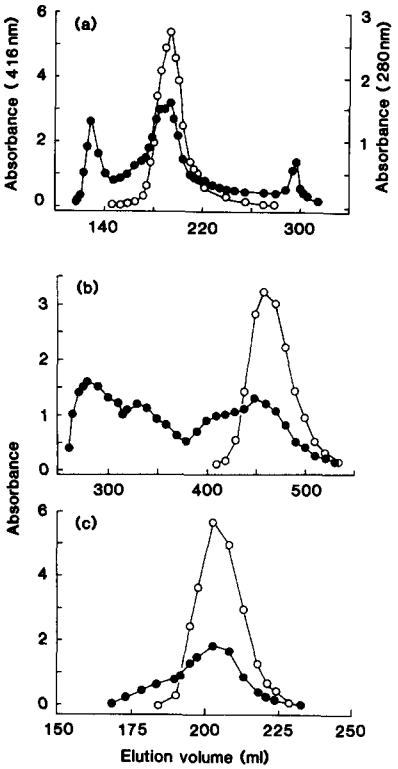

Fig. 2. (a) Elution pattern of cytochrome *c*-550 (basic) (zero-NaCl II fraction) on Sephadex G-75. ●, protein (A_{280}); ○, cytochrome *c*-550 (A_{416}); (b) and (c) purification of cytochrome *c*-550 (acidic): Gel filtration of 0.2 M NaCl fraction on Sephadex G-100 (b) followed by Gel filtration on Sephadex G-75 (c). ●, protein (A_{280}); ○, cytochrome *c*-550 (A_{416}) (see Materials and Methods for details).

Purification of cytochrome c-550 (acidic). Cytochrome *c*-550 in the 0.2 M NaCl fraction from chromatography on DEAE-Sepharose CL-6B was further purified by gel filtration on Sephadex G-100 (Fig. 2b) and then on Sephadex G-75 (Fig. 2c) with the same procedures as stated before. After each gel filtration, fractions containing cytochrome *c*-550 (with a ratio of A_{416}–A_{280} greater than 2 or 2.5) were pooled and concentrated by ultrafiltration as described before.

Polyacrylamide gel electrophoresis. SDS-polyacrylamide slab electrophoresis for both monitoring purification of proteins and determination of molecular weight of polypeptides as well as nondenaturing disc-polyacrylamide slab gel electrophoresis were as described previously [6] except that samples were boiled for 5 min before loading on to SDS-gel and the following staining procedure was used for the nondenaturing gel: fixed with fixing solution (a mixture of 28 ml 75% perchloric acid/160 ml methanol/600 ml distilled water) for 0.5 h, then stained with staining solution (0.2 g Coomassie Blue G in 400 ml fixing solution) for 1.5 h and finally destained in destaining solution (5% (v/v) acetic acid and 10% methanol in distilled water). A haem-staining procedure on SDS-gel described by Thomas et al. [8] was used.

Preparative nondenaturing disc-polyacrylamide slab gel electrophoresis was performed essentially the same as described above except that thicker gel (3 mm) and more sample (18 mg of cytochrome *c*-551) were used, and the electrophoresis was run at a constant 18 mA for 10 h at 4°C. After running the gel (which showed about six visible red bands, due to cytochrome *c*-551), it was cut into strips. The strips with cytochrome *c*-551 were individually macerated to give fine suspensions from which cytochrome *c*-551 was extracted and separated by addition of 50 mM Tris (pH 7.3), followed by filtration through Whatman No. 1 paper. The cytochrome in the solution was then concentrated by dialysis against solid polyethylene glycol. All procedures were performed at 4°C.

Preparative isoelectric focusing on Sephadex IEF. This was done as described previously [6].

Determination of isoelectric point on 1 mm poly-

acrylamide gel. This was done by flat bed electrofocusing as stated before [6] except that a broad p*I* calibration kit was employed using Pharmalyte, pH 4–6.5.

Determination of molecular weight by gel filtration. Molecular weight of cytochrome *c*-552.5 was determined on Sephadex G-100 using the same procedures as detailed before [6]. Molecular weight of cytochrome *c*-551 was measured on Sephadex G-200 and Sephacryl S-400 using ferritin (M_r 440000), catalase (M_r 210000), lactate dehydrogenase (M_r 120000) and bovine serum albumin (M_r 67000) as markers.

Spectrophotometry. Room temperature absorption spectra were done as stated previously [3]. Pyridine haemochromogen spectra were obtained in a mixture containing 10–30 μl of cytochrome and equal parts of pyridine and 0.2 M KOH to give a final volume of 1 ml after reduction with a few grains of dithionite [9]. A Pye-Unicam SP1700 spectrophotometer was used.

Haem content. The number of *c*-type haem groups per cytochrome molecule was determined by the pyridine haemochromogen method based on a millimolar extinction coefficient of 29.1 cm^{-1} at 550 nm (reduced band) [10].

Determination of iron. Iron content was measured by atomic absorption spectroscopy using a Rank Hilger Atomspek H1550. Samples (0.25 mg protein) were prepared by digesting at 100°C (15 min) in 0.5 ml $H_2SO_4 + HNO_3$ (1 + 1.8, v/v) and diluted to 2.5 ml for assay. Blanks without protein and with bovine serum albumin (which contains no iron) were used.

Proteins estimation. Protein was determined by the standard Lowry Folin method, using bovine serum albumin as a standard.

Reagents. Cytochrome *c* (horse heart III), cytochrome oxidase (bovine heart), all of the protein markers used for determination of molecular weight by gel filtration and molecular weight marker kits (MW-SDS-70L), were obtained from Sigma. Sephadex G-75, G-100, G-200, Sephacryl S-400, Phenyl-Sepharose CL-4B, Sephadex IEF, Pharmalyte (pH 2.5–5 and pH 4–6.5), low and broad PI calibration kits, Polybuffer exchanger PBE94 and Polybuffer 74-HCl were purchased from Pharmacia.

Results

Purification of cytochrome c-551. Cytochrome *c*-551 was purified by the procedures described in Materials and Methods, and the results are summarized in Table I. The final purification showed about 85% purity of the cytochrome judged by SDS-gel. Based on this, and assuming a yield of

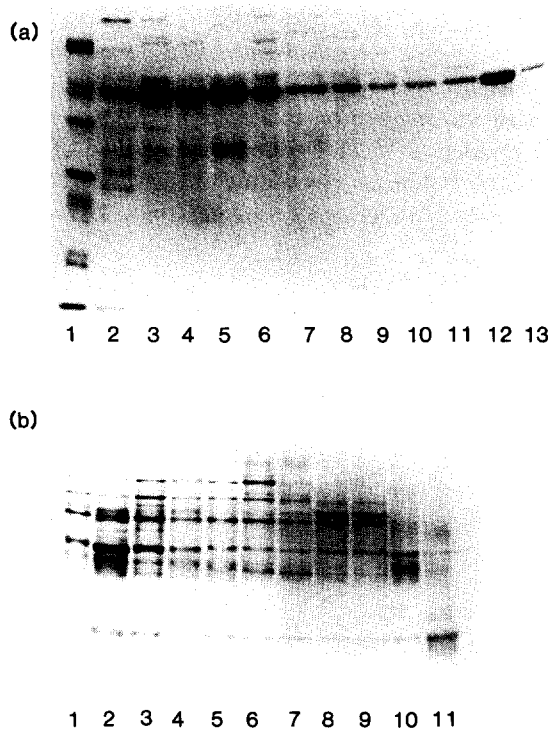

Fig. 3. (a) SDS-gel electrophoresis of the fractions in the purification of cytochrome *c*-551 and the fractions from the preparative nondenaturing gel electrophoresis. Marker proteins [1], molecular weights were indicated. Cytochrome *c*-551 fractions pooled from Sephadex G-100 (2), from Sephadex G-200 (3), from Sephacryl S-400 (4), from HIC II fraction (5), from HIC III fraction (6) and from HIC IV fraction (7). Lanes (8)–(13) were fractions from the top to the bottom band after preparative nondenaturing gel electrophoresis. Samples contained 30–80 μg protein. (b) Nondenaturing gel electrophoresis of the fractions in the purification of cytochrome *c*-551 and the fractions from the preparative nondenaturing gel electrophoresis. Cytochrome *c*-551 fraction pooled from Sephadex G-100 (1), from Sephadex G-200 (2), from HIC II fraction (3), from HIC III fraction (4), and from HIC IV fraction (5). Lanes (6)–(11) were fractions from the top band to the bottom band after the preparative nondenaturing gel electrophoresis. Samples contained 30–80 μg protein. See Materials and Methods and Results for details.

50%, cytochrome *c*-551 represented approx. 1% of the total protein in the crude extract. The purified cytochrome *c*-551 was completely free of enzyme B and sulphite : cytochrome *c* oxidoreductase activities.

Aggregation of cytochrome c-551. One of the peculiar characteristics of cytochrome *c*-551 was its polymerization as found during the purification. The cytochrome solution (G-100 fraction) was separated on hydrophobic interaction chromatography (HIC) into four major protein peaks, three of which mainly consisted of the cytochrome with some minor contaminators as shown on SDS-gel (Fig. 3). Both spectral properties and enzyme assay also confirmed that they were cytochrome *c*-551. The recovery of cytochrome *c*-551 after HIC was very low, only about 20% of the sample loaded. Hence, the procedure was not used in the large-scale purification of cytochrome *c*-551.

Whereas cytochrome *c*-551 purified after gel filtration and HIC gave only one major band on SDS-gel (Fig. 3a), the same samples showed multiple bands (about six) on nondenaturing disc-gel (Fig. 3b), which implied that they might be a sort of isoenzyme. To solve the problem, a large amount of purified cytochrome *c*-551 was run by preparative electrophoresis on nondenaturing disc-polyacrylamide gel and then removed from the gel as stated in Materials and Methods. The cytochromes from the six red bands were electrophoresed again on a nondenaturing disc-gel. Each sample continued to show four to six bands with more or less the same pattern on the gel (Fig. 3b). However, they only gave one major band on SDS-gel (Fig. 3a). The results strongly suggested that isoenzymes were not involved, otherwise only one original band would appear on the nondenaturing gel. Every fraction also showed some stimulatory activity to the thiosulpahte : cytochrome *c* oxidoreductase system.

It appeared, therefore, that cytochrome *c*-551 formed a series of aggregates from monomer to hexamer. A plot of the R_f values of the six bands on the nondenaturing disc-gel against the molecular weights of the monomer to the hexamer on semi-logarithmic paper exhibited a straight line. This finding also meant that the subunits were noncovalently bound to each other and their association and dissociation were in a constant equilibrium.

The formation of the aggregates and their equilibrium also help to explain the diverse elution pattern of cytochrome *c*-551 on HIC: thus different sizes of aggregates probably had different hydrophobic interaction strengths with the column and were eluted at different times, and the further

TABLE I

PURIFICATION OF CYTOCHROMES

Purity indices were assayed as (absorbance of reduced cytochrome at its α peak/absorbance of oxidized sample at 280 nm). Because of the presence of nucleic acid and other *c*-type cytochromes the purity index could not be assayed before the third-mentioned step.

Purification step	Cytochrome *c*-551		Cytochrome *c*-552.5		Cytochrome *c*-550 (basic)		Cytochrome *c*-550 (acidic)	
	Protein (mg)	Purity index	Protein (mg)	Purity index	Protein (mg)	Purity index	Protein (mg)	Purity index
Crude extract	39500	–	39500	–	49100	–	39500	–
Ammonium sulphate fraction (A65%)	11400	–	11400	–	12000	–	11400	–
DEAE Sepharose CL-6B								
no NaCl (II)					234	0.25		
0.2 M NaCl							660	0.18
0.35 M NaCl (I)	1150	0.13						
0.35 M NaCl (II)			962	0.45				
Sephadex G-100	355	0.24	568	0.73			125	0.51
Sephadex G-200	185	0.36						
Sephadex G-75					55	0.83	50	0.76
Chromatofocusing			378	0.77				

purification of the cytochrome was difficult simply because of the trapping of contaminating proteins during the polymerization.

Molecular weight of cytochrome c-551. The molecular weight of cytochrome *c*-551 was about 300000 estimated by gel filtration on Sephadex G-200 or on Sephacryl S-400 and 43000 (±2000) by SDS-gel. The purity of the purified cytochrome *c*-551 was about 85%, which implied that the real molecular weight of the aggregated cytochrome *c*-551 was about 260000. The figure was in good agreement with the molecular weight of the aggregate composed of six polypeptides, each of the same size (43000).

Some observations on the haem prosthetic groups of cytochrome c-551. Acid acetone (0.012 M HCl in acetone) did not extract the heam groups from cytochrome *c*-551, judged by the observation that the red colour stayed with the precipitated proteins, which indicated that the heam groups were covalently attached to the apoprotein. However, as shown in Fig. 1b, a certain amount of haem (about 10–20%), recognised by absorbance at 416 nm, was eluted at the end of the chromatography on Sephadex G-200 with very little protein (A_{280}) in these fractions. The observations accounted for the very low increase of purity index ($A_{551\ red}/A_{280\ ox}$; Table I) and might have indicated release of haem from the cytochrome. The possibility that this haem came from other sources could not be completely ruled out.

The loss of haem from cytochrome *c*-551 was also exhibited in SDS-gel electrophoresis, where the green haem band stained as described in Materials and Methods appeared at the front line. However, if the sample was treated without boiling, a certain amount of haem was seen at the band position characteristic of the apoprotein.

Cytochrome *c*-551 (G-100 fraction) was separated into two major fractions after chromatofocusing, one fraction eluted at pH 4.5–4.2 containing colourless proteins, and another eluted after addition of 1 M NaCl containing haem group as indicated by the red colour. Neither the single fractions nor a mixture of the two retained ability to stimulate the activity of the thiosulphate : cytochrome *c* oxidoreductase system. On SDS-gel the colourless fraction showed a band the same as that of native cytochrome *c*-551, and the haem-containing fraction gave several bands, one of them with the same position as that of native cytochrome *c*-551.

Spectral properties and haem content of cytochrome c-551. Fig. 4a shows the absorption spec-

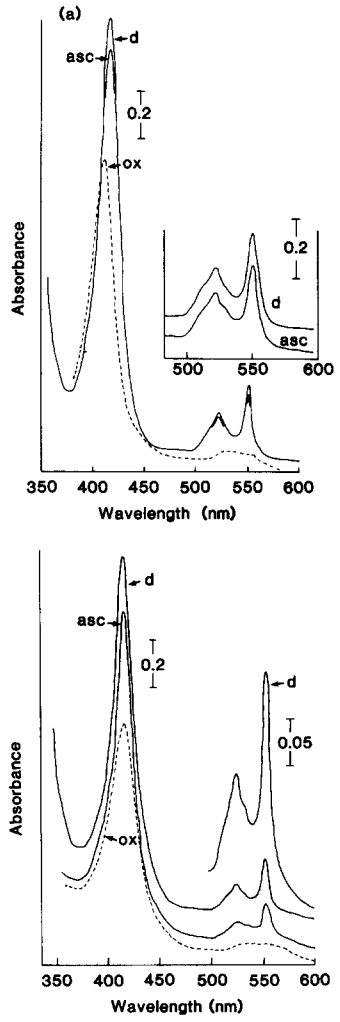

Fig. 4. (a) Absorption spectrum of cytochrome *c*-551 (- - - - - - ox) oxidized; (——— asc) ascorbate-reduced; (——— d) dithionite-reduced. 1 cm cuvette contained 1.36 mg of purified cytochrome *c*-551 in 50 mM Tris-HCl (pH 7.3) in a final volume of 1 ml. The ascorbate-reduction and dithionite-reduction were allowed to proceed for 25 and 5 min, respectively, before recording the spectra. (b) Absorption spectrum of cytochrome *c*-552.5: (- - - - - - ox), oxidized; (——— asc), ascorbate-reduced; (——— d) dithionite-reduced. 1 cm cuvette contained 0.32 mg of highly purified cytochrome *c*-552.5 in 50 mM Tris-HCl (pH 7.3) in a final volume of 1 ml. The ascorbate-reduction and dithionite-reduction were allowed to proceed for 15 and 2 min, respectively, before recording the spectra.

trum of the purified cytochrome *c*-551. Ascorbate reduced cytochrome *c*-551 to about 70% of the fully reduced state whereas dithionite completely reduced the cytochrome. The absorption maxima were 551, 552 and 418 nm in the reduced form with both reductants and 410 nm in the oxidized form. On the basis of a molecular weight of 260 000 and a purity of 85%, the millimolar extinction coefficients were calculated to be 40 cm^{-1} from $A_{551-540}$ and 64 from absolute absorbance at A_{551} (Fig. 4a).

The pyridine ferrohaemochromogen of cytochrome *c*-551 exhibited an absorption spectrum typical of *c*-type cytochrome with maxima at 550, 521 and 414 nm. By the same method of calculation, the millimolar extinction coefficient was found to be 96 cm^{-1} at 550 nm, from which the number of haem groups per cytochrome *c*-551 molecule was estimated to be 3.5. The iron content per molecule of cytochrome *c*-551 was determined to be 5.1 ± 0.8 atoms per mole [4]. From these results it can be tentatively concluded that the cytochrome contains four to five *c*-type haem groups per molecule.

PI value and the stability of cytochrome c-551. The isoelectric point of cytochrome *c*-551 was found to be pH 5.2 (± 0.3). The slow movement and the diffuse banding of the protein on the 5% polyacrylamide gel prevented precise determination. Preparative isoelectric focusing on Sephadex IEF with Pharmalyte (pH 2.5–5 or 4–6.5) failed to purify the cytochrome.

Cytochrome *c*-551 was reasonably stable when kept at 4°C or at −20°C. Thawing and freezing three times had no detectable affect on the activity of the cytochrome in the thiosulphate-oxidizing multi-enzyme system.

Purification of cytochrome c-552.5. Cytochrome *c*-552.5 was purified to homogeneity by the procedures described in Materials and Methods (Table I). Assuming a yield of 60%, the cytochrome comprised about 1.5% of the total protein of the crude extract. The purified protein gave a single diffuse band after SDS-gel electrophoresis and there may be a small amount (less than 2%) of contaminating proteins.

Purification of the cytochrome by preparative isoelectric focusing on Sephadex was unsuccessful because the cytochrome moved extremely slowly and was virtually unfocused on the gel.

Molecular weight and PI value of cytochrome c-552.5. The molecular weight of the native cytochrome *c*-552.5 was 56 000 (± 2000) as determined by gel filtration on Sephadex G-100. After SDS-gel electrophoresis a single polypeptide species was found, corresponding to a subunit M_r of about 29 000 (± 2000). Thus, the cytochrome appeared to be a dimer of subunits of equal molecular size.

The isoelectric point of cytochrome *c*-552.5 was measured by isoelectric focusing on polyacryla-

TABLE II

EFFECT OF AMOUNT AND ORDER OF ADDITION OF CYTOCHROME *c*-552.5 AND CYTOCHROME *c*-551 ON THE ACTIVITY OF THE THIOSULPHATE:CYTOCHROME *c* OXIDOREDUCTASE SYSTEM.

First addition (mg)		Second addition [a] (mg)		Activity	
cyt *c*-552.5	cyt *c*-551	cyt *c*-551	cyt *c*-552.5	nmol cyt *c* reduced per min	nmol cyt *c* reduced per min per mg
0.03		0.12		21.4	70
0.08		0.12		21.4	58
0.018		0.12		18	61
0.03		0.17		28	79
0.03		0.08		18	67
	0.12		0.03	16	52
	0.12		0.08	17	46

[a] Basic reaction mixture contained: 0.1 mg enzyme A, 0.08 mg enzyme B, 2 μmol $S_2O_3^{2-}$, 80 nmol horse heart cytochrome *c*, 45 μmol Tris-HCl (pH 7.3) and cytochrome *c*-552.5 and cytochrome *c*-551 as indicated, in a final volume of 1 ml. Cytochrome *c*-551 or *c*-552.5 was added last, as indicated. Reduction of horse heart cytochrome *c* at 550 nm was recorded spectrophotometrically at 30°C as described in Materials and Methods.

mide gel and a value of 4.8 (± 0.2) was obtained.

Spectral properties and haem content of cytochrome c-552.5. Absorption maxima of ascorbate- or dithionite-reduced cytochrome *c*-552.5 were at 552.5, 523 and 418 nm, and that of the oxidized form at 415 nm as shown in Fig. 4b. Ascorbate only partially reduced the cytochrome (about 65%), whereas dithionite completely reduced it. On the basis of an M_r of 56000, millimolar extinction coefficients for the fully reduced cytochrome were 28 at $A_{552.5-540}$ and 44 at $A_{552.5}$.

Pyridine haemochromogen of dithionite-reduced cytochrome *c*-552.5 revealed a typical *c*-type absorption spectrum with maxima at 550, 520 and 415 nm. The millimolar extinction coefficient of the band was determined to be 67, from which a value of 2.3 haem groups per molecule of cytochrome *c*-552.5 was estimated. The iron content was found to be 2.2 Fe per cytochrome *c*-552.5. Therefore, it appears that the cytochrome contains two *c*-type haem group per molecule.

Acid acetone (0.012 N HCl in acetone) did not extract the haem groups from cytochrome *c*-552.5, which indicated that they were covalently attached to the apoprotein.

Cytochrome *c*-552.5 was reasonably stable when kept at 4°C or −20°C. Thawing and freezing three times had no detectable affect on the activity of the cytochrome in the thiosulphate-oxidizing multi-enzyme system.

Studies on the involvement of cytochrome c-552.5 and cytochrome c-551 in thiosulphate oxidation. Either cytochrome could individually effect the reduction of mammalian cytochrome *c* by thiosulphate in the presence of enzymes A and B. The rate of reduction with cytochrome *c*-552.5 as catalyst showed a progressive increase while cytochrome *c*-551 gave a progressively decreasing rate, i.e., the rate was highest immediately after the start of the reaction, then slowly declined. A possibly related phenomenon (Table II) was that adding cytochrome *c*-552.5 into the reaction mixture as the last component gave lower activity than did adding cytochrome *c*-551 last. It seems that both cytochromes interacted with enzyme A and enzyme B in some way and the interaction with cytochrome *c*-551 gave lower activity, which also prevented good interaction between cytochrome *c*-552.5 and the two enzymes, producing lower activity if cytochrome *c*-551 was added before cytochrome *c*-552.5. Table II also demonstrates that the amount of cytochrome *c*-552.5 promoting activity of the multi-enzyme system was about five times less than for cytochrome *c*-551.

Although either cytochrome *c*-552.5 or cytochrome *c*-551 with enzyme A and enzyme B catalysed the reduction of mammalian cytochrome by thiosulphate, the oxidation of thiosulphate to sulphate was complete only in the presence of all four compounds (Table III), together with cytochrome oxidase and horse-heart cytochrome. The complete oxidation of thiosulphate was also confirmed by experiments with ^{35}S-labelled thiosulphate to measure the formation of sulphate. The reaction mixture without cytochrome *c*-552.5 gave no reaction at all. However, the mixture without cytochrome *c*-551 did show very slow oxygen uptake and incomplete oxidation of thiosulphate with a ratio of O_2 to $S_2O_3^{2-}$ about 1. This finding was interesting in that it showed a partial reaction, but the low oxidation rate did not enable us to separate and identify the intermediates or the product(s) of the partial reaction using ^{35}S-labelled thiosulphate.

TABLE III

OXIDATION OF THIOSULPHATE BY THE PURIFIED COMPONENTS

The experiment was done in an oxygen electrode as described before [3]. Standard reaction mixture contained: 0.4 mg enzyme A, 0.2 mg enzyme B, 0.09 mg cytochrome *c*-552.5, 0.25 mg cytochrome *c*-551, 0.5 mg horse heart cytochrome *c*, 5 units bovine heart cytochrome oxidase and 50 mM (pH 7.3) Tris-HCl to a final volume of 1 ml. $Na_2S_2O_3$ (100 nmol precisely) was added to start the reaction. All of the components were highly purified, and there was no sulphite:cytochrome *c* oxidoreductase activity at all in the enzyme B and the cytochrome *c*-551 fractions. All of the figures were means of three experimental results. A detailed description of the involvement of sulphite:cytochrome *c* oxidoreductase in the thiosulphate oxidation is in preparation (Lu and Kelly, unpublished data).

Reaction mixture	Rate of O_2 uptake (nmol $O_2 \cdot min^{-1}$)	$S_2O_3^{2-}$ added (nmol)	O_2 uptake (nmol)
Complete	22	100	204
minus cytochrome *c*-552.5	no reaction		
minus cytochrome *c*-551	1.6	100	88
minus Enzyme A	no reaction		
minus Enzyme B	no reaction		

The different observations obtained with the spectrophotometric method and with oxygen electrode methods were probably due to (a) the former method being much more sensitive (over 100 times in the terms of number of electrons transported for a response of one division on the chart paper) than the latter; (b) the reaction conditions being not the same; for example, the intensive stirring used in the oxygen electrode might affect or disrupt interactions between components of the complex which were essential to the reaction.

Table IV shows that cytochrome *c*-552.5 and cytochrome *c*-551 as well as cytochrome *c*-550 (basic) were gradually reduced by thiosulphate in the presence of enzymes A and B. The reduction of cytochrome *c*-550 (basic) was complete, as addition of dithionite did not increase the absorbance, whereas the reduction of cytochrome *c*-552.5 or cytochrome *c*-551 was only partial, about 60% and 70% of fully reduced form, respectively. Cytochrome *c*-551 was nearly fully reduced by the supplementary addition of sulphite: cytochrome *c* oxidoreductase. Reduced cytochrome *c*-551 was slowly and partially reoxidized. Adding sulphite and sulphite: cytochrome *c* oxidoreductase did not reduce cytochrome *c*-552.5 any further, although it was completely reduced by dithionite. Horse heart cytochrome *c* also was fully reduced by thiosulphate in the presence of enzyme A and enzyme B although the rate was very slow. These observations were in good agreement with the finding that cytochrome *c*-552.5 and cytochrome *c*-551 were partially reduced by ascorbate and fully reduced by dithionite (Fig. 4a and b), which indicated that these two cytochromes probably contain two redox centres: a high-potential one, reduced by ascorbate and thiosulphate, and a low-potential one, whose reduction was effected by dithionite or thiosulphate in the case of cytochrome *c*-551. These findings were confirmed by redox potential titration experiments (Lu, Poole and Kelly, unpublished data).

Purification and properties of cytochrome c-550 (basic). Cytochrome *c*-550 (basic) was purified to about 80% purity (Table I). On the basis of 80% purity and supposing a yield of 50% the cyto-

TABLE IV

REDUCTION OF *c*-TYPE CYTOCHROMES BY ENZYMES A AND B WITH THIOSULPHATE AND THE EFFECT OF SUBSEQUENT ADDITION OF SULPHITE: CYTOCHROME *c* OXIDOREDUCTASE, SULPHITE AND DITHIONITE

The reaction mixtured contained: 0.06 mg enzyme A, 0.05 mg enzyme B, 2 μmol $S_2O_3^{2-}$, 40 μmol Tris (pH 7.3) and cytochrome as indicated in a total volume of 1 ml. The reaction was started by adding enzyme B and the increase in the absorbance at the α-band of the cytochrome being assayed was followed spectrophotometrically. Both enzyme A and B were necessary for the reduction of the *c*-type cytochromes.

Cytochrome	Concentration (μM)	Change in absorbance at α-band maximum for each cytochrome					Ratio of B to E [c]
		A No addition (background)	B After addition of $Na_2S_2O_3$	C Rate (maximum) (ΔAα-band per min)	D Effect of [a] addition of 'sulphite oxidase' to B (ΔAα-band)	E Effect of dithionite (ΔAα-band)	
Cytochrome *c*-552.5	11.4	0.23	0.394	0.06	0.394 [b]	0.51 [d]	0.59
Cytochrome *c*-551	11.5	0.18	0.38	0.05	0.75	0.8 [d]	0.67
Cytochrome *c*-550 (basic)	25		0.45	0.02	–	0.45 [e]	
Cytochrome *c*-550 (acidic)	50		0	0			
Horse heart Cytochrome *c*	13		0.4	0.003	–	0.4 [e]	

[a] Sulphite: cytochrome *c* oxidoreductase (10 μg).
[b] Na_2SO_3 (2 μmol) added with the sulphite oxidase.
[c] Background values deducted from B and E.
[d] Dithionite added after treatment D.
[e] Dithionite added after treatment B.

chrome comprised about 0.3% protein of the crude extract. The M_r was determined by SDS-gel electrophoresis to be 15000. The isoelectric point of the cytochrome was about (or higher than) pH 8 by the fact that the band for the cytochrome appeared at the top of the denaturing gel after electrophoresis at pH 8.8.

The cytochrome was completely reduced by ascorbate or dithionite with absorption maxima at 550, 521 and 415.

The absorption maxima of the pyridine ferrohaemochromogen of cytochrome *c*-550 (basic) were at 550, 521, 414 nm. On the basis of the M_r and a purity of 80% the millimolar extinction coefficient was found to be 26 (A_{550}), which was very close to that of mammalian cytochrome *c*. It thus apparently contained one haem group per mole.

Cytochrome *c*-550 (basic) was slowly but completely reduced by thiosulphate in the presence of enzymes A and B (Table IV), although the cytochrome did not accelerate the oxidation of thiosulphate by the multi-enzyme system when measured either spectrophotometrically or polarographically.

Purification and properties of cytochrome c-550 (acidic). Cytochrome *c*-550 (acidic) was purified to about 70% (Table I). The cytochrome was not precipitated by ammonium sulphate, so ultrafiltration was used to concentrate the cytochrome.

The M_r was estimated by SDS-gel electrophoresis to be 14000, but was about 29000 by gel filtration on Sephadex G-75. This presumably indicated the formation of a dimer composed of two 14000 M_r subunits. The isoelectric point was found at pH 5 (± 0.2) by isoelectric focusing on polyacrylamide gel.

The cytochrome was completely reduced by ascorbate or dithionite, giving absorption maxima at 550, 552 and 415 nm. Absorption maxima of its pyridine ferrohaemochromogen were at 550, 520 and 414 nm. The millimolar extinction coefficient was 36 (A_{550}), indicating one haem group per mole.

The cytochrome was not reduced by thiosulphate in the presence of enzyme A and enzyme B (Table IV), and had no effect on the thiosulphate-oxidizing system.

Discussion

The novel redox carriers or effectors of the thiosulphate-oxidizing system, cytochrome *c*-552.5 and cytochrome *c*-551, have been purified from *Thiobacillus* A_2. They are termed *c*-type cytochromes mainly because of their spectral and haem group properties, although in many aspects such as high molecular weight, presence of several subunits and haem groups and, most of all, the presence of two redox centres, they are quite different from the *c*-type cytochromes previously found in living organisms. The two cytochromes comprise about 2.5% of the total crude extract protein, which indicates their importance. It is also worth noting that this thiosulphate-oxidizing multi-enzyme system (two enzymes and two cytochromes) represents some 4% of the crude extract protein or about 3.2% of the total protein of the organism.

From the facts that cytochrome *c*-552.5 and cytochrome *c*-551 are necessary components for the thiosulphate-oxidizing multi-enzyme system and both of them can easily be reduced by thiosulphate in the presence of enzyme A and enzyme B, the two cytochromes appear to be the primary electron carriers linking oxidation by the two enzymes to the next components of the electron transport chain, which could be membrane-bound or associated *c*-type cytochrome(s).

Furthermore, since the thiosulphate oxidizing activity of the multi-enzyme system in the presence of both cytochrome *c*-552.5 and cytochrome *c*-551 is much higher than that in the presence of only one of them, cytochrome *c*-552.5 and cytochrome *c*-551 also appear to function as effectors. The mechanism of this effect might be (a) molecular interactions between the two cytochromes or between them and enzymes A and B, or (b) as thiosulphate oxidation involves the transfer of four pairs of electrons, one or other cytochrome might accept electron specifically from particular oxidation reactions, such as sulphane-sulphur oxidation steps or from sulphonate sulphur, so that if only one of them is present in the reaction mixture some partial product(s) of the reaction would accumulate, which would prevent or retard further reaction (Table III). There is some evidence to support point (b) showing that cytochrome *c*-551 might be a redox carrier for electrons from

sulphonate-sulphur, as follows: (i) from the purification results, cytochrome *c*-551 is very closely associated with sulphite : cytochrome *c* oxidoreductase, and the total separation of the two causes dramatic loss (around 90%) of the activity of sulphite : cytochrome *c* oxidoreductase; (ii) the low activity was variably increased by 15–30% in several experiments by the addition of a small amount of cytochrome *c*-551 and only about 10% by that of cytochrome *c*-552.5 (Lu and Kelly, unpublished data); (iii) the low redox potential centre of cytochrome *c*-551 is partially reduced by sulphite : cytochrome *c* oxidoreductase. If *c*-551 is indeed involved in the oxidation of the sulphonate group, it may be deduced that cytochrome *c*-552.5 could serve as a direct acceptor of electrons from sulphane-sulphur, although direct evidence is still lacking. The partial oxidation of thiosulphate catalysed by cytochrome *c*-552.5, the two enzymes and the electron chain (Table III), could mean that intermediates may accumulate and would be available for detection and identification if the reaction rate can be improved in the future. Recently, a cytochrome *c*-551 was purified from another facultative thiobacillus, *Thiobacillus novellus* [12,13]. This cytochrome was shown to be tightly bound to the sulphite : cytochrome *c* oxidoreductase, and M_r 23 000 (by SDS-gel electrophoresis) or about half that of the cytochrome *c*-551 from *Thiobacillus* A_2. It is interesting to note that the two *c*-551 cytochromes show some similarities, such as being being involved in sulphite oxidation, having the same p*I* value (5.2) and more or less the same spectral properties at room or liquid nitrogen temperatures and of their pyridine ferrohaemochromogens. Only two *c*-type cytochromes (the other being a small cytochrome *c*-550) have, however, been identified and purified from *T. novellus* so far.

Two distinct *c*-551 cytochromes involved in thiosulphate metabolism were also purified and characterized from two thiosulphate-oxidizing *Chlorobium* strains [14,15]. One of them had an M_r of 45 000 (by SDS-gel electrophoresis), two haem groups per molecule and was shown to be reduced by thiosulphate in the presence of a thiosulphate-cytochrome *c*-551 reductase [16]. Coincidentally, this reductase had an M_r of 80 000 which may be compared to a combined M_r of about 80 000 for enzymes A and B of *Thiobacillus* A_2 [6]. The reduction rate was greatly enhanced on addition of cytochrome *c*-555, a flavin-cytochrome of M_r 50 000. Although the system in the photosynthetic bacteria may not be directly comparable with that in chemolithotrophs, these findings do suggest an important and general role in the oxidation of thiosulphate for these novel *c*-type cytochromes, which are characterized by their large molecular weights, low p*I* values and often containing more than one haem group per protein.

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